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## **Community Composition of Bacterial Biofilms Formed on Simple Soil Based Bioelectro- chemical Cell Anodes and Cathodes**

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## Abstract:

Microbial fuel cells (MFC), as bioelectrochemical systems, hold promise as a sustainable source of energy for use in novel environments and settings. Although electrode biofilms, both anode and cathode, are critical to the production of power in these systems, the taxonomies of the biofilms that form are not fully understood. The specific objectives of the current study were to classify the bacteria that enriched onto fuel cell electrodes, with three biogeochemically distinct surface soils serving as the inocula. Following 1000 hours of incubation under saturated soil conditions, the community composition of the anode and cathode bacterial biofilms was quantified by culture, fatty acid profile (FA), and terminal restriction fragment length polymorphisms (TRFLP). The three soils produced open circuit voltages of between 60 and 120 mV, but only one soil maintained voltage under resistance (60 mV at 10,000  $\Omega$ ). Fatty acid profiling identified differences among anode, cathode, and bulk soil microbial communities, specifically a greater relative abundance of terminally branched saturated FA in the bulk soils and a greater relative abundance of monounsaturated and cyclopropane FA on electrode surfaces. Bacteria cultured from both the anode and cathode biofilms included species of Actinobacteria; however, only the anode biofilms produced species of Firmicutes and Proteobacteria. Analysis of TRFLP profiles putatively identified a diversity of bacteria in the biofilms recovered from both electrode surfaces. The most predominant organisms detected were the  $\alpha$ - and  $\beta$ -Proteobacteria, specifically the Rhizobiales, Rhodobacterales, and Burkholderiales. Although the detection of similar community fingerprints on both anodes and cathodes was not anticipated, the diversity of organisms putatively identified indicated a complexity that would support a coupled nitrogen cycle, one capable of facilitating the transfer of electrons to the soil cell anodes and from the soil cell cathodes.

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## Preface

The work was performed by David B. Ringelberg, Karen L. Foley, and Dr. Charles M. Reynolds (Biogeochemical Sciences Branch, Terrence M Sobecki, Chief), U.S. Army Engineer Research and Development Center—Cold Regions Research and Engineering Laboratory (ERDC-CRREL). At the time of publication, Dr. Justin Berman was Chief of the Research and Engineering Division. The Deputy Director of ERDC-CRREL was Dr. Lance Hansen, and the Director was Dr. Robert Davis.

The use of trade, product, or firm names in this report is for descriptive purposes only and does not imply endorsement by the U.S. Government. The tests described and the resulting data presented herein, unless otherwise noted, were obtained from research conducted under the Installation Technology Program of the U.S. Army Corps of Engineers by the USAERDC. Permission was granted by the Chief of Engineers to publish this information. The findings of this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

COL Kevin J. Wilson was the Commander and Executive Director of ERDC, and Dr. Jeffery P. Holland was the Director.

## 1 Introduction

In 1912, Michael Potter identified an electrical effect that accompanied the microbial decomposition of organic matter. Although the ability of bacteria to catalyze the conversion of organic matter into electricity has been known for over 75 years, the use of bacteria in fuel cells to generate appreciable electrical power is a more recent occurrence. In 2003, Chaudhuri and Lovely were able to show that a novel bacterium, *Rhodoferax ferrireducans*, could transfer electrons directly to a graphite electrode while oxidizing a carbon substrate. The organism derived energy for growth and produced electricity from the coupled process. Rabaey et al. (2004) then demonstrated that microbial fuel cell (MFC) anodes can enrich for anodophylic communities that form biofilms capable of self-mediating electron transfer, resulting in stable and long term power densities. Soluble redox mediators or electron shuttles have been identified that facilitate electron transfer to fuel cell anodes (Rabaey et al. 2005) and, as it turns out, the electron shuttles produced by one bacterium can be utilized by other species of anodic bacteria (Pham et al. 2008). Certain bacteria have also been shown to produce conductive pilus-like structures, nanowires that enable a direct transfer of electrons from cell membrane to the anode surface (Reguera et al. 2005). That bacteria are capable of colonizing MFC electrodes and that they possess the molecular traits to enable electron transfer to these electrodes has been established. Research is now focusing on the breadth of anodophylic bacteria and microbes that exist in nature and into the factors that regulate electron transfer mechanisms.

The large percentage of studies examining bioelectrochemical system (BES) performance have focused on sewage sludge and wastewater as bacterial inocula, where anaerobic growth within the anodic chamber produces the electrons that when reduced at the cathode result in lower wastewater CODs (Alterman et al. 2006). Recent studies have begun looking into the nature of these biofilms in terms of community taxonomy and the activity related to electron transfer and power production. A number of studies have identified the Proteobacteria, including members of the alpha, beta, gamma, and delta classes, as being prominent anodophylic organisms (Kim et al. 2004; Phung et al. 2004; Kim et al. 2007; Chung and Okabe 2009). Molecular analyses, specifically of the 16S rRNA eubacterial gene, have also identified the presence of Firmicutes and Actinobacteria

on wastewater enriched anodes (Chung and Okabe 2009; Phung et al. 2004, respectively).

Of growing interest is the performance of MFC under aerobic conditions. Traditional BES used a two-chamber system where bacteria in an anode compartment were separated from the cathode compartment by a polymeric proton exchange membrane (PEM). Many of these MFC used aqueous cathodes with oxygen supplied via air sparging. Advances in design demonstrated that BES employing air-cathodes and the absence of a PEM were still efficient and effective at generating electricity (Liu and Logan 2004). In addition, these forms of BES were less costly and amenable to aerobic applications, such as in riverine systems. Acknowledgement of military as well as commercial uses of MFC for continued surveillance systems or for powering environmental sensors has lead to developments in the miniaturization of BES. Novel designs have been used to minimize anode cathode distances and to take advantage of 3D electrode materials to significantly increase electrode surface areas (Shantaram et al. 2005; Ringeisen et al. 2006).

Although the interaction between an anode and a microbial biofilm is a critical component of any bioelectrochemical system, microbe facilitated electron transfer off of fuel cell cathodes can also play an integral role. The reduction of O<sub>2</sub> at the cathode is often a significant bottleneck in BES efficiency and chemical catalysts, such as platinum, pyrolyzed iron, phthalocyanine, and cobalt tetramethylphenylporphrin, have all been used successfully to facilitate O<sub>2</sub> reduction. However, Clauwaert et al. (2007) showed that bacteria can also facilitate the O<sub>2</sub> reduction on the cathode surface and the formation of a cathode microbial biofilm, i.e., a biocathode, has appeal in terms of BES performance in terms of cost savings and improved power sustainability.

Microbial fuel cells as bioelectrochemical systems hold promise as a sustainable source of energy for use in novel ways and in novel environments. Metagenomics studies of anodic biofilms have and will likely continue to shed new light on the breadth of community functional dynamics that BES systems are capable of supporting (Liu et al. 2010). In this study we describe the molecular trait of three different soil based bioelectrochemical systems. We provide a polyphasic taxonomic description of the anodic and cathodic bacterial biofilms and identify common characteristics for use as a base for further development and refinement of BES for use in terrestrial environments.

## 2 Materials and Methods

### 2.1 Soil characteristics

Characteristics of the three soils examined in the study are provided in Table 1. Two of the study soils, abbreviated FG and FW, were collected from areas outside of Anchorage, AK, and the third soil, abbreviated CRL, was collected from the Cold Regions Research and Engineering Laboratory, Hanover, NH. The soils were all air dried, sieved to 4.75 mm (FG and FW soils) and 2.00 mm (CRL soil) and stored at room temperature. Prior to introduction into the bioelectrochemical cells, each soil was brought to 0.33 bar moisture content and incubated at room temperature for 72 hours.

Table 1. Geochemical characteristics are provided for each soil used to fill the anodic chamber of the three test fuel cells.

		Fairbanks, AK USA	Fairbanks, AK USA	Hanover, NH USA
		FW	FG	CRL
pH		7.8	5.6	6.5
soluble salts	mmhos/cm	0.10	0.13	0.18
CEC	meq/100g	16.6	2.3	2.7
TOC/O.M.	%	1.4	11.1	2.5
NO <sub>3</sub> -N	ppm	6.0	7.8	2.4
NH <sub>4</sub> -N	ppm	6.3	<3.9	5.1
N/C ratio	NH <sub>4</sub> /TOC	4.6	0.4	2.0
P	ppm	4.4	0.8	2.2
K	ppm	39	16	57
Ca	ppm	1345	294	533
Mg	ppm	97	97	40
Fe	ppm	nd	3.5	0.6
Mn	ppm	nd	8.1	1.2
Cu	ppm	nd	0.0	0.0
Zn	ppm	3.8	0.2	1.2
Texture		sandy loam	silty loam	sandy loam
sand	%	49	29	49
silt	%	46	60	44
clay	%	5	12	7
nd = not detected				

## 2.2 Bioelectrochemical cells

A schematic of the bioelectrochemical cells is provided in Figure 1. Each cell consisted of an anodic chamber filled with soil and separated from the surrounding cathodic chamber by a cation exchange membrane (CMI-7000S, Membranes International, Inc.). The cation exchange membrane was sealed to the bottom of the glass jar with paraffin wax (~2 cm height). Each chamber, anode and cathode, contained a graphite electrode of equal dimensions, 3 mm deep  $\times$  5 mm wide  $\times$  100 mm long. The anodic chamber was loaded with ~5 g of soil, tamped, the electrode placed in the center, another 5 g of soil added and tamped around the electrode, etc., until the chamber was filled to within 1–2 mm of the top. The cathodic chamber was filled with deionized sterile water and continuously sparged with air. The electrode was then suspended in the water between the glass jar and the CEM. A small portion of the anodic soil, ~0.1 g, was added to the cathodic water as inocula. Voltage was measured across the two electrodes every hour using a digital multimeter (Kiehly Instruments) either as an open voltage or across a 10,000  $\Omega$  resistor.

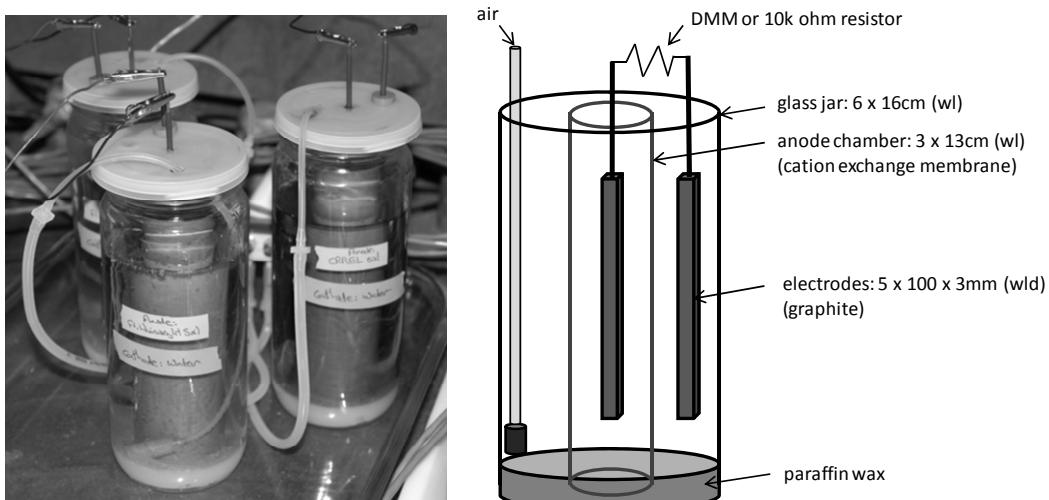


Figure 1. Actual soil cells with associated schematics.

## 2.3 Sample collection

Each BES cell was sampled to obtain taxonomic descriptions of the electrode biofilms and the surrounding media, i.e., soil or water. Although in some instances, sufficient material was not obtained for a particular assay, the sampling scheme outlined below was followed for each cell. Anode electrodes were first pulled from the soil columns and tapped gently to remove loosely attached soil clumps. The electrodes were then dipped in

sterile milli-Q water to recover the attached soils (AS), which were then collected by centrifugation at 8400 rpm for 20 minutes. The supernatant was decanted and the soils resuspended in 2 mL of sterile water for subsequent assay of lipids (0.8 mL), nucleic acids (0.8 mL), and culturable cells (0.4 mL). Biofilms remaining on electrodes were then recovered by swabbing each side with separate 5- × 5-mm sections of autoclaved (15 min. at 5.7 kPa) glass fiber filter paper (GF/A Whatman). One third of each swab was removed, suspended in 2 mL of sterile water and vortexed vigorously before removing 0.2-mL aliquots for cell culture. The remaining swabs were then directly assayed for either lipids or nucleic acids. Anode soils were separated into three sections, top, middle, and bottom, and mixed using a spatula before aliquots were removed to assay for lipids (2 g) and nucleic acids (0.2 g). Both the catolyte solution and the cathode electrodes were then sampled. Cells were recovered from the catholyte by centrifuging 30 mL at 9400 rpm for 20 minutes. The cell pellets were then resuspended in 1.6 mL of sterile milli-Q water and 0.8 mL was removed for determining lipid content and 0.8 mL for determining nucleic acid content. Cathode electrodes were processed in the same manner as the anode electrodes, excluding the step of dipping the electrode in sterile milli-Q water. Culture for bacterial isolation was not performed on either the catholyte solution or the anode soils.

## 2.4 Sample analysis

### 2.4.1 Culturable bacteria

Serial dilutions of each 0.2–0.4 mL aliquot was spread plated onto PTYG agar [5 g Peptone, 5 g tryptone, 5 g yeast extract, 10 g glucose, 0.6 g  $MgSO_4 \cdot 7H_2O$ , 0.06 g  $CaCl_2 \cdot 2H_2O$  per 1 L water at pH 7.0] and R2A agar (Difco, Becton Dickinson). Following 48 hours of incubation at 25°C, single colonies were picked and re-streaked for isolation. Isolated colonies were then identified by fatty acid profiles using the Microbial Identification System (MIDI Inc.) using Sherlock v4.0 software.

### 2.4.2 Lipid assay

In situ fatty acid profiles were obtained from the bulk anode soils, the cathode waters, and the anode and cathode electrode swabs. Briefly, total lipids were extracted from sample materials in a volume of chloroform:methanol:phosphate buffer (50 mM  $PO_4$  at pH 7.4) in the ratios of 1:2:0.8 (v:v:v) (White and Ringelberg 1998). Soils, 2 g, and filter papers

were extracted in 3.8 mL of the extractant. For the catholyte and loosely attached soils, 0.8 mL of the suspensions was used in place of the 50 mM PO<sub>4</sub> buffer. Following phase separation and recovery of total lipids, a strong acid methanolysis was used to form fatty acid methyl esters (FAME), which were then recovered in a volume of hexane:chloroform (4:1, v:v). The FAME were then further separated, quantified, and identified by gas chromatography/mass spectrometry. Methyl nonadecanoate, 50 pmol  $\mu$ L<sup>-1</sup> was used as an internal standard and individual fatty acids were expressed on a mole per gram basis. The relative molar percentage of each fatty acid was used for statistical comparisons.

#### **2.4.3 Terminal restriction fragment length polymorphisms (TRFLP)**

Total nucleic acids were recovered from the anode filter paper swabs using the PowerPlant™ DNA Isolation Kit (Mo Bio Laboratories). A Precellys 24 homogenizer (Bertin Technologies) was used to facilitate cell lysis. The primer pair 27F-FAM (5' – FAM- aga gtt tga tcc tgg ctc ag – 3') and 926R (5' – ccg tca att cct ttr agt tt – 3') was used to amplify a 16S rDNA fragment of approximately 915 bp in length. Approximately 100–200 ng of the amplicon was then purified on a QiaQuick PCR purification spin column (Qiagen) before digestion with 5 U of the restriction enzymes HhaI, MspI and RsaI at 37°C for 3 hours. Enzyme activity was stopped by heating at 60°C for 30 minutes. The digested amplicons were then desalting on Qiaquick nucleotide removal spin columns (Qiagen) and the concentrations adjusted to approximately 100 ng  $\mu$ L<sup>-1</sup>. Purified fragments were then separated by capillary electrophoresis (15-s injection time at 15 kV, 15 kV electrophoretic voltage for 45 min.) on an Applied Biosystem 310 genetic analyzer. Fragments were sized against an x-rhodamine labeled 1000 bp ladder (MapMarker 1000, Bioventures, Inc.) using the local southern method within Peak Scanner software (v1, Applied Biosystems). Resulting TRF profiles were post-processed following the outline of Dunbar et al. (2000). TRF area percentages were then imported into the MICA 3 T-RFLP analysis (PAT+) program to perform in silico digests of the ribosomal database (release 10 update 12) of good quality 16S rRNA fragments (Shyu et al. 2007). Identifications resulting from each digest were compared using the on-line software program VENNY (Oliveros 2007) to produce putative identifications at the phylum, class, and order levels.

#### **2.4.4 Statistical analysis**

Principal components analysis was performed on arcsine square root transformed fatty acid molar percentages (Statistica v8). Means comparisons was by Tukey honest significant difference via a post hoc analysis and significance assumed at a  $p < 0.05$ .

### 3 Results and Discussion

The primary purpose of the simple soil cells was to facilitate anodic colonization by the extant soil bacteria. To date, there have been few efforts investigating soils as potential anode inocula (Schamphelaire et al. 2008; Schamphelaire et al. 2010; Ishii et al. 2008a,b; Jiang et al. 2010). The intent of this study was to augment these investigations by providing a polyphasic description of biofilms enriched onto graphite electrodes. Soils are non-ideal environments for MFC operation primarily because of the presence of the preferred electron acceptor oxygen and to existing constraints to mass transport. To lessen the negative impacts of these conditions, we brought the soils to near saturation with water. By doing so we hoped to facilitate electron transfer via soluble redox mediators and bacterial access to available soluble nutrients in the soils. In addition, we postulated that the near saturated soil conditions would favor biofilm formation and, once formed, these biofilms would exhibit a redox gradient that was increasingly negative from the outside in (Bishop and Yu 1999).

As the cells were not flow through and there was no active transport of carbon or nutrients to the anode electrode surface, we assumed that only the soil microbes in proximity to the anode electrode would be enriched to form an anode biofilm. By filling the anode chamber with sieved and homogenized soil mixtures, impacts from natural soil heterogeneity in terms of microbial diversity were reduced. By collecting the loosely attached anode soil in addition to the direct anode biofilm, we intended to recover both the enriched and the enriching bacterial populations. With regards to the cathodic chamber, no salt was added to the catholyte solution, which is typically done to facilitate electron reduction at the cathode. This choice was made to minimize the likelihood of selective enrichment of halophilic organisms. Although the construction and imposed experimental conditions of each soil cell were non-ideal in terms of power production, the potential for electricity was not fully impeded and obtaining an electrical current was crucial to establishing that the enriched electrode biofilms were at least partially electrogenic in nature.

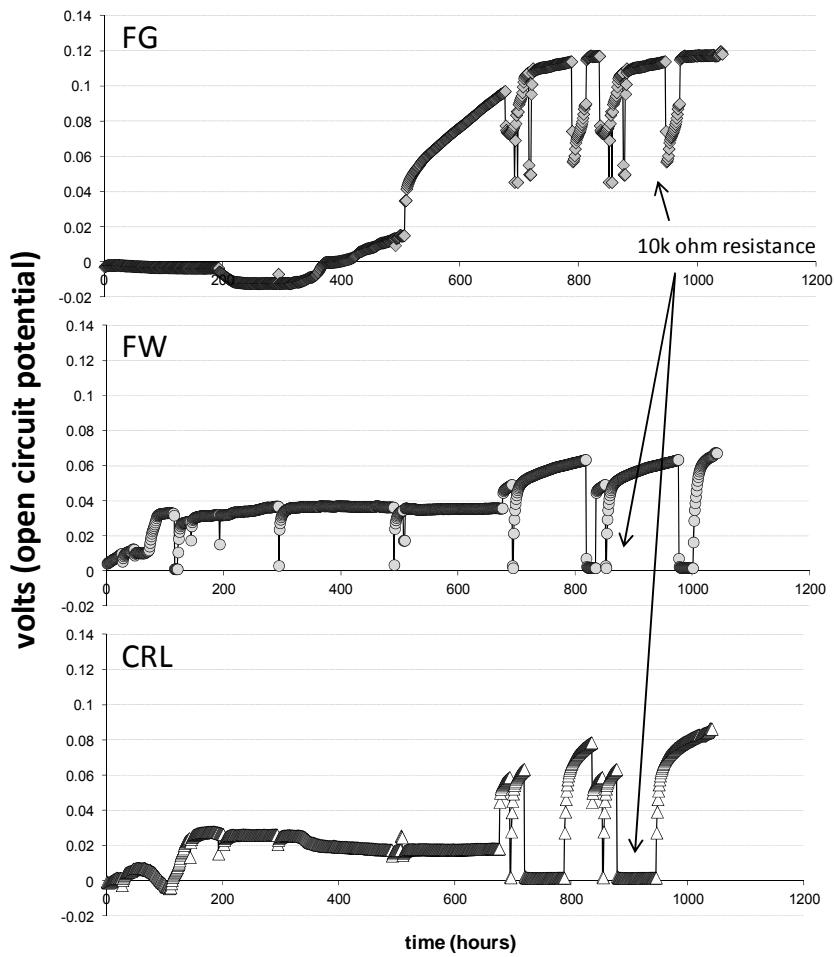


Figure 2. Voltage, open circuit potential, measured from the three soil cells over a 100 hour period. The sharp voltage declines indicate points in time where the output was measured across a 10-k $\Omega$  resistor

Results showed all three fuel cells produced electricity. Open circuit potentials (OCP) of between 60 and 120 mV were recorded following an incubation period of 42 days (Fig. 2). Although these voltages were 6 to 110 times less than those recorded from a more efficient soil seeded MFC (Jiang et al. 2010), the increasing and sustained voltages suggested successful bacterial colonization of the embedded graphite anodes. Placing a small load, 10,000  $\Omega$ , onto each soil cell had a large effect on the voltage output from each cell, with only the FG cell exhibiting a measurable current of approximately 5  $\mu$ A. This soil differed substantially from the other two soils in terms of total organic carbon content, which was five times greater, and iron and manganese content, which were five to eight times greater, respectively (Table 1). In addition, the pH of the FG soil, at 5.6, was substantially lower than that of either the CRL, 6.5, or FW soil, 7.8. Biffinger et al. (2008) examined the effect of pH on an aerobic miniature MFC and found

that pH not only affected biofilm biomass but, more importantly, the production of a soluble redox mediator, riboflavin. In this study, anode microbial biomass recovered from the FG electrode was greater than that recovered from the other two cells, 2.4 nmol FA versus 0.9 to 1.4 nmol (Table 2), and this may explain the 1.5- to 2-fold greater OCP observed with the FG cell. However, pH and specific metabolic functions, discussed below, could equally have influenced biofilm development and influenced electron transport processes. The acquired data only indicate that there was successful colonization of the electrodes in all three soils and that viable mechanisms must have existed for active transfer of electrons to and from the electrodes. Although the mechanisms of electron transfer were not specifically investigated, the bacterial taxonomy of the enriched electrode biofilms was examined in detail.

As indicated above, the recovery of fatty acids from the electrode surfaces of all three soil cells indicated presence of viable microbial biofilms. Fatty acid profiles can provide insight into microbial community abundance and composition and serve as a quantitative measure of community change over time or due to perturbation (Vestal and White 1989). As inocula, all three soils contained a substantial microbial biomass, with little differences being observed top to bottom (Table 2). For the CRL and FW soil cells, the amount of fatty acid recovered from the soils was comparable, averaging 25 to 33 nmol g<sup>-1</sup> of soil, respectively, which was slightly greater than that observed in the FG soil, 19 nmol g<sup>-1</sup>. Assuming 1 pmol of fatty acid is quivalent to  $2.5 \times 10^4$  bacterial cells (Balkwill et al. 1988), we find that then the cell density for the three soils varied from 5 to  $8 \times 10^8$  cells per gram of soil.

### 3.1 Anode biofilms

Although eukaryotic biomarkers were detected on the anode surfaces in all three test cells, this biomass composed less than 10% of the total microbial biomass recovered (Table 2). This result suggests that electron transfer at the anodes was mediated principally by prokaryotes. We assumed that any prokaryotic biomass that was successful in colonizing an anode surface would show a different composition than that present in the surround soil matrix owing to the stark differences in available substrates. Principal components analysis of the prokaryotic fatty acid profiles indicated that differences existed between the compositions of soil and anode bacterial communities (Fig. 3). Factor variable-correlations for the 1<sup>st</sup> principal component identified the shift or difference in community composition,

soil to anode, to be characterized by increased relative percentages of monounsaturated and normal saturated fatty acids (Table 3). A means comparison of specific prokaryotic FA biomarkers indicated that the normal saturated and monounsaturated FA were significantly different ( $p < 0.05$ ), with the anode biofilms showing the greater relative molar percentages (Fig. 4).

**Table 2. Relative molar percentages and absolute abundances of prokaryotic and eukaryotic fatty acid (FA) biomarkers detected in the soils, top middle and bottom fractions, on the electrodes, biofilm and attached soil, and in the catholyte of the three test cells are provided. Biomarker abbreviations are described in the accompanying key.**

Prokaryote FA biomarkers	CRL soil cell						FW soil cell						FG soil cell								
	soils			anodic chamber		cathodic chamber			soils			anodic chamber		cathodic chamber			soils			anodic chamber	
	top	middle	bottom	biofilm	attached soil	catholyte	biofilm	top	middle	bottom	biofilm	attached soil	catholyte	biofilm	top	middle	bottom	biofilm	attached soil	catholyte	biofilm
scNSat %	20	21	19		53	41	25	19	19	18	63	65		25	11	11	11	53	18	18	14
anteiso-odd %	6	5	5		4	2	1	6	5	5	3	6		1	2	2	2	3	3	4	
iso-odd & even %	12	11	11		4	2	1	7	6	7	2	2		2	3	4	4	6	5	6	3
Cyclo %	3	3	3		4	8	2	3	2	2	4	1		7	1	1	2	1	3	14	6
Mono %	13	14	13		26	36	24	11	11	11	17	17		38	5	6	5	32	15	39	46
BrMono %	4	4	3		2	7	3	3	3	3	2	1		5	1	1	1	2	3	6	5
MidBrSat %	5	5	4		1	0	4	3	3	3	0	0		4	0	1	1	0	1	1	0
Hopene %	1	1	2		0	0	0	2	2	1	0	0		6	0	0	1	0	1	2	3
dimeAcetal %	0	0	0		0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0
$\beta$ -hydroxy %	1	1	1		1	0	0	1	1	1	0	1		1	1	1	1	0	1	2	0
Total prokaryotic %	64	66	62		94	96	60	54	52	50	91	93		89	24	27	27	98	50	90	83
soil biomass (nmol g <sup>-1</sup> )	15.53	16.21	16.32					14.11	21.93	14.81				3.63	3.83	7.43					
electrode biomass (nmol)					0.88		2.08							4.11				2.33	16.54		18.46
catholyte biomass (nmol ml <sup>-1</sup> )						1.23														11.73	
Eukaryote FA biomarkers																					
Poly %	2	2	2		2	1	2	1	2	1	2	2		5	0	0	0	1	1	4	2
dioic %	26	25	27		2	0	37	36	33	38	2	1		0	75	72	70	1	41	1	12
sterol %	3	3	4		0	0	0	5	8	5	4	0		0	0	0	0	1	0	0	
meoxy %	1	1	1		1	3	0	1	1	1	0	0		3	0	0	1	0	1	4	3
alcohol %	2	2	2		1	1	1	2	3	3	1	5		1	0	0	1	1	5	1	1
Total eukaryotic %	34	32	36		6	4	40	45	47	49	9	7		10	76	73	72	2	49	9	17
soil biomass (nmol g <sup>-1</sup> )	8.18	7.97	9.51					11.89	19.51	14.38				11.56	10.43	19.57					
electrode biomass (nmol)					0.05		1.40				0.13	0.03		0.47				0.05	16.34		3.88
catholyte biomass (nmol ml <sup>-1</sup> )						0.05														1.19	
total microbial soil biomass (nmol g <sup>-1</sup> )	24.11	24.54	26.22		0.93		3.47		26.30	41.95	29.59	1.41	0.46	4.60		15.24	14.35	27.21	2.38	33.06	22.36
total microbial electrode biomass (nmol)																				13.10	
total microbial catholyte biomass (nmol ml <sup>-1</sup> )							1.28														

<sup>1</sup> values represent the relative molar percentage each biomarker contributes to overall sample profile.

Key:	
abbrv.	description
scNSat	short chain normal saturated (n12:0 - n18:0)
TerBrSat	terminally branched saturated
iso-odd	iso-odd branched saturated
iso-even	iso-even branched saturated
anteiso-odd	anteiso-odd branched saturates
Mono	monounsaturated
Cyclo	cyclopropyl
BrMono	methyl branched monounsaturated
MidBrSat	mid-chain methyl branched saturated
Hopene	hopanoids
dimeAcetal	dimethyl acetals
$\beta$ -hydroxy	$\beta$ -hydroxy saturated (n10:0 - n18:0)
Poly	polysaturated
dioic	di-carboxylated
sterol	sterols & stanols
meoxy	methoxy and oxo fatty acids
alcohol	fatty alcohols
	functional group

A broad diversity of bacteria has previously been identified in anode biofilms. Proteobacteria, including members of the alpha, beta, gamma, and delta classes, have all been identified as anodophylic organisms when enriched from a variety of inocula that include wastewaters, sewage sludge, and sediments, (Kim et al. 2004; Phung et al. 2004; Kim et al. 2007; Chung and Okabe 2009). Molecular analysis of 16S rRNA genes have resulted in the identification of Firmicutes (typically Gram-positive) and Actinobacteria (often Gram-variable) on the anodes of fuel cells inoculated with wastewaters (Chung and Okabe 2009; Phung et al. 2004, respectively). Results of the fatty acid analyses performed in this study largely agree

with these findings in terms of the types of bacteria detected and expand the data to delineate the community of bacteria present in the adjacent soil from that directly composing the anode biofilms.

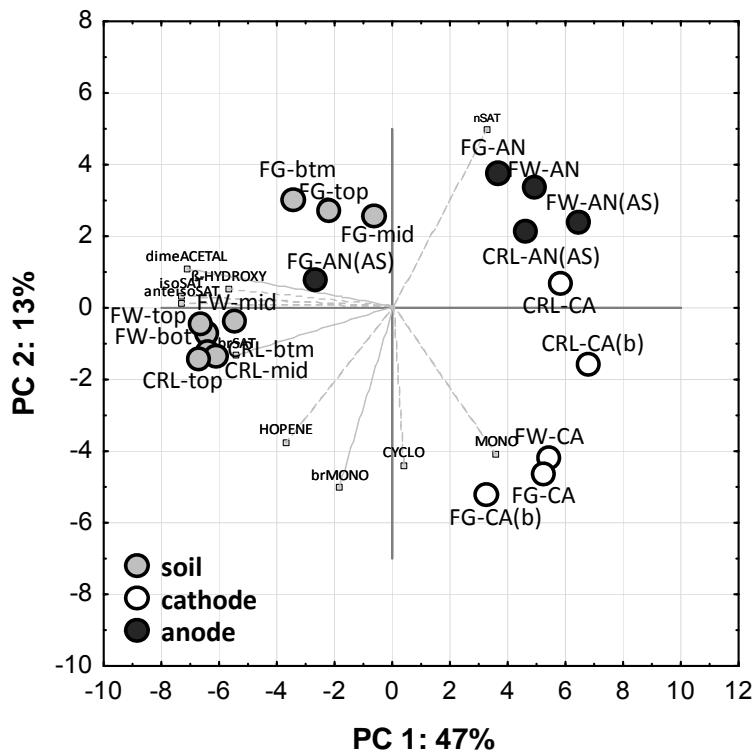
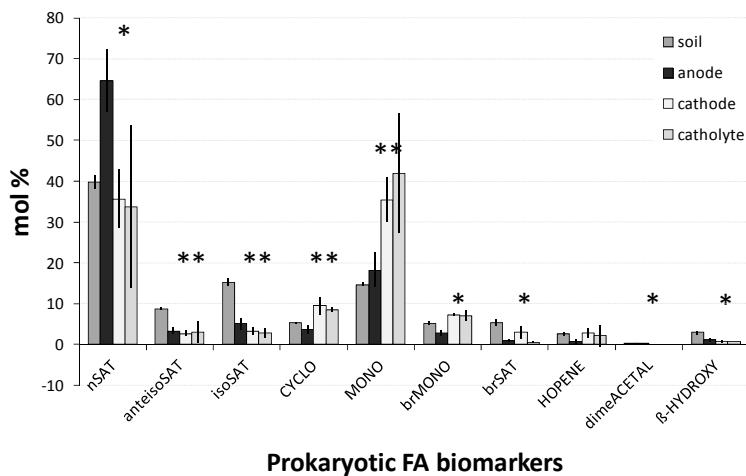


Figure 3. 2D plot illustrating results from the extraction of the 1<sup>st</sup> two principal components from a dataset comprised of arcsine square root transformed fatty acid molar percentages. Fatty acid profiles recovered from the bulk soil, top, bottom (btm), and middle (mid) fractions, the cathodes, catholyte (b) and cathode biofilm (CA) samples, and the anodes, attached soil (AS) and anode biofilms (AN) for each of the three soil cells, FG, FW, and CRL. The superimposed bi-plot illustrates factor variable correlations (loadings) associated with the first two principal components. The fatty acid functional group abbreviations are described in Table 2.



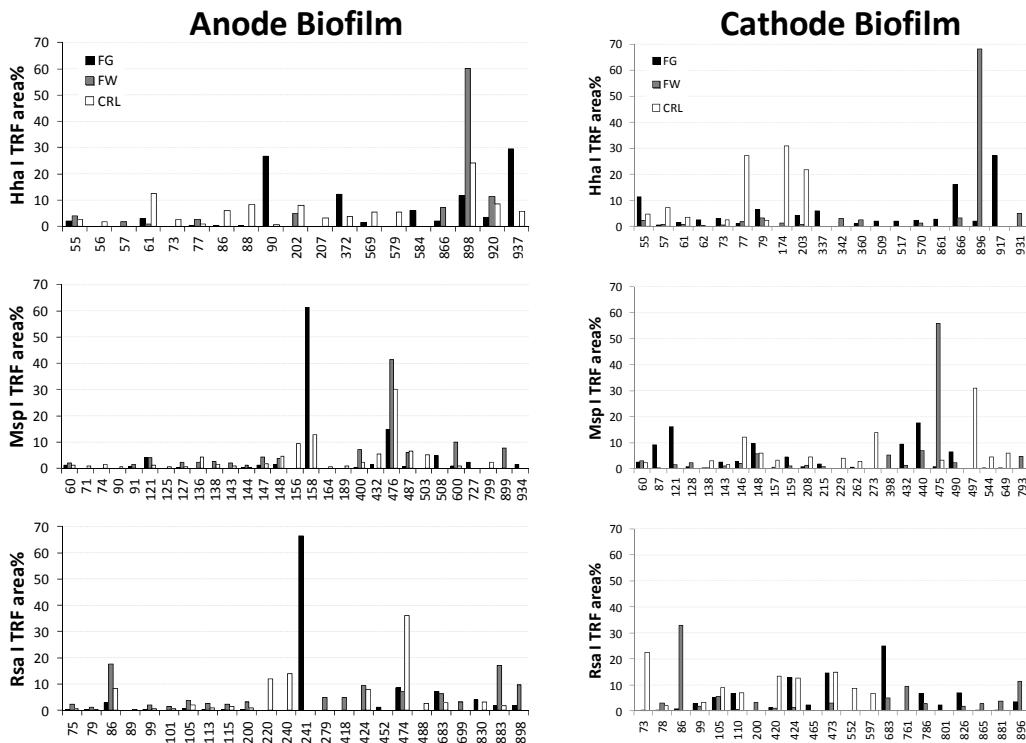
**Figure 4.** A means comparison of fatty acid methyl ester (FAME) structural class molar percentages (mol%) across the averaged soil profiles (top, middle and bottom, n=9), anode attached soils and biofilms (n=5), cathode biofilms (n=3), and catholyte (n=2). A Tukey HSD post hoc comparison of means identified means that differed significantly at  $p < 0.05$  (\*) or  $p < 0.01$  (\*\*).

**Table 3.** Factor variable correlations (loadings) between prokaryotic fatty acid (FA) profiles and the 1<sup>st</sup> principal component that resulted following the principal components analysis of soil, electrode, and catholyte arcsine square root transformed fatty acid molar percentages.

Prokaryotic FA biomarkers	PC1
	factor-variable correlations
monounsaturate	6.20
normal saturate	5.42
cyclopropyl	0.54
CH <sub>4</sub> branched monounsaturate	-3.13
mid-chain CH <sub>4</sub> branched saturate	-8.58
hopene	-10.66
β-hydroxy saturate & unsaturate	-11.84
iso CH <sub>4</sub> branched saturate	-13.91
anteiso CH <sub>4</sub> branched saturate	-15.00

Examination of the 16S rRNA gene through terminal restriction fragment length polymorphisms (TRFLP) provided a greater resolution from which anode community similarities and dissimilarities could be determined (Fig. 5). In principle, each terminal restriction fragment (TRF) generated

from a given restriction digest should represent a unique species of bacteria (Marsh 1999). Although this assumption does not always hold true (Blackwood et al. 2007), TRFLP profiles have been successfully used to fingerprint a targeted community and to make comparisons between communities (for example, Kuehl et al. 2005). With the assumption that each identified TRF represents a unique organism, phylotype richness can be estimated from the number of TRF detected. Results from the anode biofilms indicated a similar richness across the three soil cells, with the CRL anode biofilm showing a slightly greater richness, 58 total detected TRF, compared to both the FG and FW cell anodes, 44 and 40 total TRF, respectively. A direct comparison of anode biofilm TRF profiles for the three soil cells indicated both commonalities and differences. Specific TRF, for example *Msp* I 476 bp, were detected in all three biofilms, suggested the enrichment of a common organism from all three of the soil inocula. Other TRF, for example *Rsa* I 220 bp, were enriched from a single soil cell. Results indicate that the bacterial taxonomy of the three anode biofilms differed as a result of the starting inocula, but also that the enriched biofilms shared some common characteristics.



**Figure 5. Recovered and retained terminal restriction fragments (TRF) for each of the three soil cells (FG, FW, and CRL) following three restriction digests (Hha I, Msp I, and Rsa I) of bacterial 16S rRNA amplicons of electrode (anode and cathode) biofilm DNA. Each TRF is identified by size as base pairs (bp) and by relative area percentage (area%).**

In silico digests of the ribosomal database were then performed to assign putative identifications to the recovered TRF (Table 4). Of the identified genera, the N<sub>2</sub>-fixing Rhizobiales were well represented among all three anode biofilms (Fig. 6). The presence of diazotrophs on anode surfaces has also been reported by others. Ishii et al. (2008a) identified a filamentous anode biofilm community composed of a Rhizobiales that developed from rice paddy soil as an inoculum. A *Mesorhizobium* sp. was observed, in situ, by Sukkasem et al. (2008) when examining the anode biofilm of a single chamber air cathode MFC inoculated with wastewater. Saito et al. (2010) recently observed that anodes with an N/C ratio of 0.7 achieved a greater power density than those with an N/C ratio of 3.8, suggesting that the presence of excess nitrogen, which is inhibitory to bacterial nitrogen fixation, limited the growth or activity of diazotrophs on the anode surface. In this study, the soil cell producing the greatest voltage, FG, also showed the lowest N/C ratio, ~10 times lower (Table 1). In addition to the Rhizobiales, all three biofilms showed evidence for the presence of species of burkholderiales, and rhodobacterales. Studies have identified β-proteobacteria, if not Burkholderia sp. specifically, and other α-Proteobacteria such as the Rhodobacterales as members of anode biofilm communities (Phung et al. 2004; Chung and Okabe 2009; Lee et al. 2010). Results from this study indicate that anode colonization by diazotrophic organisms had occurred and that these organisms, Rhizobiales and/or Rhodobacterales likely played an electrogenic role in each of the three soil cells. However, these were not the only organisms putatively identified and, in agreement with the fatty acid analysis, the detection of Firmicutes, Cyanobacteria and δ-Proteobacteria suggests a complexity to the anode bacterial communities.

Both the CRL and FW soil cell anodes yielded putative TRF bacterial identifications that were unique to the particular test cells. Unique to the FW cell anodes was the putative identification of cyanobacteria. Pisciotta et al. (2010) recently demonstrated a light-dependent electrogenic activity in variety of cyanobacteriathat appeared to be fundamentally different from the known electrogenic activity of bacteria. Unique to the CRL cell anode was the putative identification of myxococcales. The δ-proteobacteria have been identified on the anodes of a number of MFC systems, and in particular those operated with wastewaters and under anaerobic conditions. Even though the soil cells in this study were not operated anaerobically, the addition of water to near saturation would have favored the formation of an-aerobic niches, such as those that occur at surface-biofilm interface (Bishop and Yu 1999). Although differences in soil chemistry likely affected the

development of the anode biofilm communities, it is believed that the community composition is also a reflection of the electrogenic nature of the biofilm (Wrighton et al. 2010).

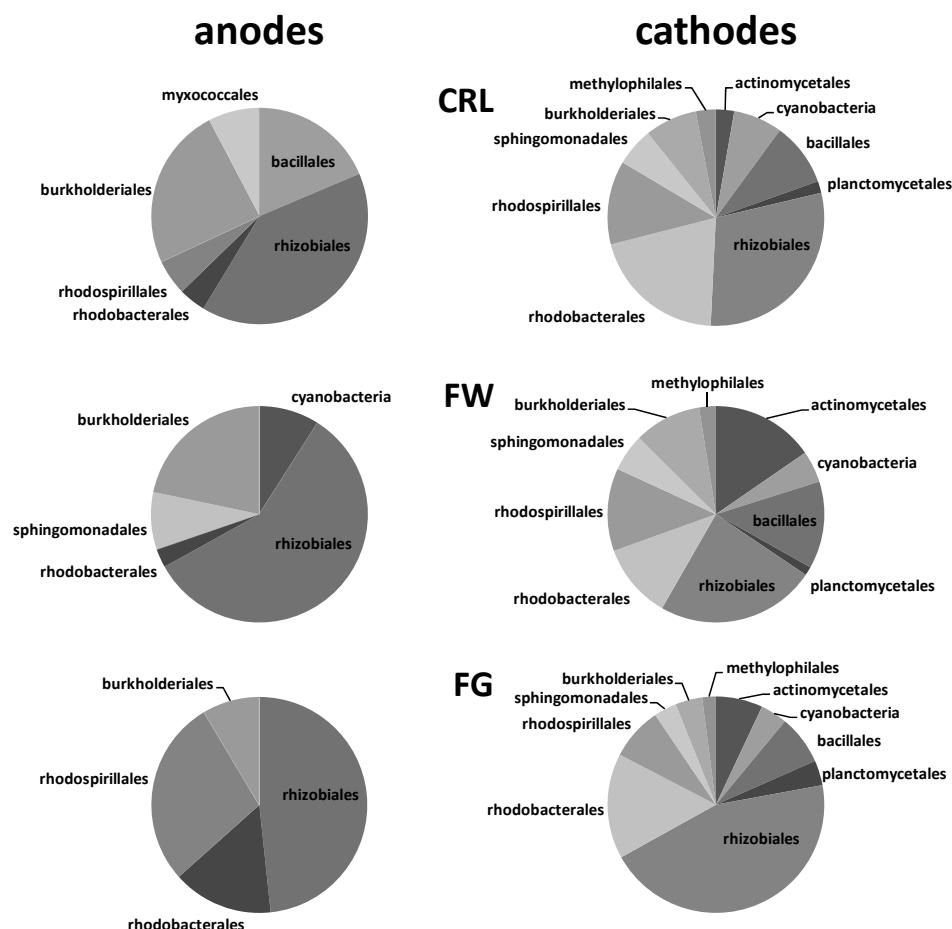


Figure 6. Pie charts of each of the three soil cell anode and cathode biofilm terminal restriction fragments that could be putatively identified through an *in silico* digest of the ribosomal database. The identified organisms and associated area percentages are provided in Table 4.

Table 4. Putative identifications for retained terminal restriction fragments of amplified 16S rRNA DNA recovered from the anodes and cathodes of the three test cells following the in silico digest of the ribosomal database. Sample TRF that could be matched to an organism are provided along with the normalized (to 100%) relative area percentages.

In addition to the above described in situ analyses, we also performed enrichments and isolation to obtain single organisms for future study. Those bacteria susceptible to enrichment, isolation, and identifications are provided in Table 5. All three anodes yielded Burkholderiales, Rhodobacterales, and Bacillales, all in agreement with the TRFLP results. In addition, a large number of Actinomycetals were isolated from the anode biofilms. Unfortunately, the lack of enrichment of Rhizobiales may reflect a bias attributable to media formulations and/or culture conditions. Never-the-less, the in situ community analyses by FA indicated an enrichment in monounsaturated FA onto anode surfaces, which are representative of Gram-negative bacteria such as the Proteobacteria. The in situ analysis by TRFLP also putatively identified the presence of Proteobacteria on the anode surfaces of all three test cells. Lastly, species of  $\alpha$ - and  $\beta$ -Proteobacteria were enriched and isolated from the anode surfaces. Voltage output from the FG soil cell was approximately 2-fold greater than that of either the FW or CRL cells. Under the assumption that the electrogenic nature of the biofilms is reflected by the extant community composition, then the FG anode surface should show a distinguishing characteristic.

The relative percentage of monounsaturated FA from the FG biofilm, 32%, was slightly greater than that extracted from either the FW, 17%, or the CRL, 26%, anode biofilms (Table 2). The relative percentage of FG biofilm TRF putatively identified as being from  $\alpha$ -Proteobacteria, 92%, was far greater than those recovered from the FW or CRL biofilms, 69 and 49%, respectively (Table 4). In contrast, TRF putatively identified as belonging to  $\beta$ -Proteobacteria were detected at only 8% in the FG biofilm compared to 22 and 32% in the FW and CRL biofilms. A similar result was also seen with the percentage of TRF putatively identified as belonging to Firmicutes. The results are consistent in identifying the  $\alpha$ -Proteobacteria as substantial colonizers of graphite anodes and suggest that their abundance is directly related to the electrogenic capacity of the anodes.

**Table 5.** Enriched, isolated, and identified bacteria recovered from the anode and cathode biofilms of each of the three test cells. Bacteria were identified by fatty acid profile using the Sherlock identification system.

soil	isolate ID	electrode	Identification	index	phylum	class	order
FG	i17	anode(attached)	Kocuria-kristinae	0.697	Actinobacteria	Actinobacteria	Actinomycetales
	i18	anode(attached)	Bacillus-cereus-GC subgroup A	0.823	Firmicute	Bacilli	Bacillales
	i26	anode(loose)	Kocuria-kristinae	0.724	Actinobacteria	Actinobacteria	Actinomycetales
	i25	anode(loose)	Bacillus-myoides-GC subgroup A (Bacillus cereus group)	0.651	Firmicute	Bacilli	Bacillales
	i27	anode(loose)	Paenibacillus-polymyxa (Bacillus)	0.746	Firmicute	Bacilli	Bacillales
	i28	anode(loose)	Brevundimonas-vesicularis (Pseudomonas vesicularis)	0.309	Proteobacteria	$\alpha$ -proteobacteria	Caulobacterales
	i23	cathode	Arthrobacter-globiformis-GC subgroup A (some 48h)	0.565	Actinobacteria	Actinobacteria	Actinomycetales
	i21	cathode	Kocuria-kristinae	0.706	Actinobacteria	Actinobacteria	Actinomycetales
	i22	cathode	Micrococcus-luteus-GC subgroup B (includes ATCC 9341)	0.619	Actinobacteria	Actinobacteria	Actinomycetales
FW	i39	anode(attached)	Arthrobacter-oxydans	0.646	Actinobacteria	Actinobacteria	Actinomycetales
	i35	anode(attached)	Rhodococcus-erythropolis/R.globerulus/N.globerula	0.952	Actinobacteria	Actinobacteria	Actinomycetales
	i36	anode(attached)	Bacillus-cereus-GC subgroup A	0.715	Firmicute	Bacilli	Bacillales
	i31	anode(attached)	Bacillus-megaterium-GC subgroup A	0.908	Firmicute	Bacilli	Bacillales
	i40	anode(attached)	Brevibacillus-parabrevis-GC subgroup B	0.460	Firmicute	Bacilli	Bacillales
	i32	anode(attached)	Paracoccus-denitrificans	0.461	Proteobacteria	$\alpha$ -proteobacteria	Rhodobacterales
	i52	anode(loose)	Arthrobacter-oxydans	0.847	Actinobacteria	Actinobacteria	Actinomycetales
	i48	anode(loose)	Kocuria-kristinae	0.880	Actinobacteria	Actinobacteria	Actinomycetales
	i45	anode(loose)	Bacillus-megaterium-GC subgroup A	0.803	Firmicute	Bacilli	Bacillales
	i51	anode(loose)	Brevibacillus-parabrevis-GC subgroup B	0.769	Firmicute	Bacilli	Bacillales
	i47	anode(loose)	Janthinobacterium-lividum	0.836	Proteobacteria	$\beta$ -proteobacteria	Burkholderiales
	i49	anode(loose)	Paracoccus-denitrificans	0.619	Proteobacteria	$\alpha$ -proteobacteria	Rhodobacterales
	i43	cathode	Microbacterium-flavescens (Aureobacterium, Arthrobacter)	0.059	Actinobacteria	Actinobacteria	Actinomycetales
	i44a,b	cathode	Micrococcus-luteus-GC subgroup A	0.222	Actinobacteria	Actinobacteria	Actinomycetales
	i42	cathode	Stenotrophomonas-maltophilia (Xanthomonas, Pseudomonas)	0.165	Proteobacteria	?-proteobacteria	Xanthomonadales
CRL	i1	anode(attached)	Bacillus-cereus-GC subgroup A	0.352	Firmicute	Bacilli	Bacillales
	i5	anode(attached)	Ralstonia-eutropha (Alcaligenes eutrophus)	0.946	Proteobacteria	$\beta$ -proteobacteria	Burkholderiales
	i16	anode(loose)	Bacillus-cereus-GC subgroup A	0.610	Firmicute	Bacilli	Bacillales
	i12	cathode	Arthrobacter-globiformis-GC subgroup A (some 48h)	0.366	Actinobacteria	Actinobacteria	Actinomycetales
	i10	cathode	Kocuria-kristinae	0.684	Actinobacteria	Actinobacteria	Actinomycetales
	i9	cathode	Microbacterium-barkeri (Aureobacterium, Corynebacterium)	0.654	Actinobacteria	Actinobacteria	Actinomycetales

### 3.2 Cathode Biofilms

Biocathodes facilitate the efficient reduction of oxygen within the cathodic chamber and the composition of cathode biofilms can also reflect the performance of an MFC (Clauwaert et al. 2007). Wrighton et al. (2010) determined that bacterial community structure of cathode biofilms corresponded to MFC performance and that  $\alpha$ - and  $\gamma$ -Proteobacteria, and Firmicutes, made up a large percentage of the cathode community. In contrast to this study, the cathode communities described by Wrighton et al. (2010) were initiated from wastewater treatment sludge and propagated in a minimal nutrient media. Considering that no nutrient supplements were added to the cathodic chamber in this study, other than the small aliquot of soil, the presence of any biofilm at all suggests a selective enrichment of bacteria capable of using the cathode, at least in part, as a source of electrons.

Fatty acid profiles and abundances in the catholyte and cathode biofilms were found to be similar, which is attributed to the restrictive nature of the

growth media present in the cathode chamber (Fig. 3 and 4). Cathode biofilms also showed a diversity of prokaryotic FA, which was composed primarily of monounsaturated FA and to lesser extents by terminally branched and mid-chain branched saturated FA, a similar result to that observed with the anode biofilms. The diversity of FA detected on the cathodes suggests some complexity to the composition of these biofilm communities, which was similar in nature to that described for the anode biofilms. However, the two electrode biofilms did differ substantially in the amount of eukaryotic biomass detected, which constituted between 10 and 40% of total cathode biofilm microbial biomass (Table 2). Of the eukaryotic functional groups detected, dioic fatty acids, which are indicative of some yeasts, were prominent in both the CRL and FG soil cells. Yeasts have been shown to mediate electron transfer to MFC anodes and are considered by some to be ideal electrogenic organisms owing to their growth rates and broad spectrum for substrates (Gunawardena et al. 2008; Ganguli and Dunn 2009; Haslett et al. 2011). Neither molecular nor enrichments/isolations were performed to further identify any cathode eukaryotes; however, the presumed presence of higher organisms on the cathodes suggests that these organisms may have played a role in mediating electron transfer from the electrodes.

Bacterial communities on the cathodes were also further evaluated through TRFLP analysis of amplified 16S rRNA gene fragments. Unexpectedly, TRF recovered from the biocathodes indicated a phylotype richness that was 14–20% greater than that observed on the respective anodes in the FG and FW cells, but 45% lower in the CRL cell (Fig. 5). The formation of similar, but not identical, biofilms on both anodes and cathodes was not anticipated. We assumed that the prevalence of carbon substrates present in the soils surrounding the anode electrodes would produce a biofilm considerably different from that forming on the cathodes. The finding of similar communities on both electrodes suggests a specific influence of the graphite surfaces on biofilm formation. Although the catholyte was not augmented with nutrients or carbon substrates other than that present in the initial inoculums, the results of both the FA and TRFLP assays indicate that conditions within the cathodic chamber were sufficient to support the growth of a biofilm as or more complex than that observed on the corresponding soil embedded anodes.

A number of recovered TRF could also be putatively assigned to bacterial species (Table 4). Rhizobiales, Rhodobacterales, Rhodospirillales,

Burkholderiales, and Bacillales were prominent in each of the three cathode biofilms. These are the same orders that were found to be prominent in the anode biofilms. In addition, Actinomycetales were putatively identified in each of three biofilms and these were the only order of bacteria, other than a single  $\gamma$ -Proteobacteria, that were susceptible to enrichment and isolation from the cathode biofilms (Table 5). Of the cultured Actinomycetales, species of Arthobacter and Micrococcus have been identified as electrogenic and effectively used as anode inocula (Hou et al. 2011; Choi et al. 2007). Although Wrighton et al. (2010) did identify the presence of actinobacteria on a biocathode, these organisms were not found to be predominant, a finding similar to this study. These non-spore forming cocci are both chemoorganotrophic and usually grow on simple media. Their ability to utilize a variety of substrates for metabolism likely enabled their colonization of the soil cell cathodes.

The presence of diazotrophs on both the anodes and cathodes may suggest a dual role for these organisms. As discussed above, Saito et al. (2010) suggested that lower N/C ratios at the anode could translate into greater MFC power densities. We also observed a greater voltage output in a soil cell that exhibited a low N/C ratio. However, N/C ratios at the cathodes should have been similar for all three cells because of the consistency of bulk media type and the minimal amount, 0.1 g, of soil added. As the catholyte was not amended with organic nitrogen, the ability to fix atmospheric nitrogen would have been advantageous to organisms within the cathode biofilm. Nitrogen fixing activity at the cathode, as evidenced by the putative identification of Rhizobiales, could have made a product for subsequent nitrification and denitrification. Although nitrifiers were not specifically identified from the in silico digests, potential denitrifying populations were. Similar to the result of Wrighton et al. (2010), members of the Burkholderiales and Bacillales were prominent on the cathodes of all three test cells examined in this study. It is possible that the diversity of organisms putatively identified on the cathodes describes a complex community that supports a coupled nitrogen cycle, one that ultimately facilitates the reduction of oxygen at the electrode surface.

## 4 Summary and Conclusion

The simple cells proved to be sufficient test vessels for examining the development of anodophyllic and cathodophyllic soil organisms. The recovery of a microbial biomass from both anodes and cathodes and the identification of community differences between the inocula and the biofilms that formed indicated that a selective enrichment had occurred. The measurement of an electrical output suggested that these enriched biofilms were, at least partially, electrogenic in nature. Polyphasic analysis of anode biofilm taxonomy identified an enrichment of Proteobacteria, specifically  $\alpha$ -Proteobacteria. In situ analyses indicated a predominance of Rhizobiales and differences in soil geochemistry, specifically the N/C ratios, suggested a correlation between the abundance of these organisms and the electrogenic capacity of the anode biofilms. In the cathode chamber, the diversity in situ biomarkers detected in the electrode biofilms suggested some complexity to the composition of these biofilm communities that was not only similar in nature to that of the anode biofilms, but also of greater phylotype richness. One distinguishing characteristic of the cathode biofilms was an abundance of an eukaryotic biomass, specifically one attributed to yeasts. Results of this study identified the existence of complex biofilms on both anodes and cathodes. The diversity and types of organisms identified on the electrode surfaces in this study were similar to the findings of others, and as a whole these results suggest that the turnover of nitrogen at the electrode surfaces, anode or cathode, can play a significant role in MFC function.

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# REPORT DOCUMENTATION PAGE

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14. ABSTRACT  Microbial fuel cells (MFC), as bioelectrochemical systems, hold promise as a sustainable source of energy for use in novel environments and settings. Although electrode biofilms, both anode and cathode, are critical to the production of power in these systems, the taxonomies of the biofilms that form are not fully understood. The specific objectives of the current study were to classify the bacteria that enriched onto fuel cell electrodes, with three biogeographically distinct surface soils serving as the inocula. Following 1000 hours of incubation under saturated soil conditions, the community composition of the anode and cathode bacterial biofilms was quantified by culture, fatty acid profile (FA), and terminal restriction fragment length polymorphisms (TRFLP). The three soils produced open circuit voltages of between 60 and 120 mV, but only one soil maintained voltage under resistance (60 mV at 10,000 $\Omega$ ). Fatty acid profiling identified differences among anode, cathode, and bulk soil microbial communities, specifically a greater relative abundance of terminally branched saturated FA in the bulk soils and a greater relative abundance of monounsaturated and cyclopropane FA on electrode surfaces. Bacteria cultured from both the anode and cathode biofilms included species of Actinobacteria; however, only the anode biofilms produced species of Firmicutes and Proteobacteria. Analysis of TRFLP profiles putatively identified a diversity of bacteria in the biofilms recovered from both electrode surfaces. The most predominant organisms detected were the $\alpha$ - and $\beta$ -Proteobacteria, specifically the Rhizobiales, Rhodobacterales, and Burkholderiales. Although the detection of similar community fingerprints on both anodes and cathodes was not anticipated, the diversity of organisms putatively identified indicated a complexity that would support a coupled nitrogen cycle, one capable of facilitating the transfer of electrons to the soil cell anodes and from the soil cell cathodes.								
15. SUBJECT TERMS Bioelectrochemical cell Fatty acid profile			Microbial community composition TRFLP					
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